# X-ray microscopy with compact pulsed sources

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## ABSTRACT

X-ray microscopy inherently possesses characteristics complementary to optical and electron microscopy. Short wavelength x-ray radiation, especially in the so-called, 'water window' (2.5 – 5 nm), permits a twenty-fold improvement in spatial resolution over optical microscopy while preserving a depth of field large enough (~100 nm) to image whole biological specimens in their natural state. Whereas electron microscopy can access atomic-scale resolution, this can only be applied to biological and medical specimens at the expense of detrimental preparation procedures (staining, drying, fixing, sectioning, etc.) that preclude real-time analysis of structural changes in living organisms. We describe progress being made in an x-ray imaging technology that provides high-resolution (~10 nm) single frame x-ray images of *in-vitro* specimens captured in a time sufficiently short that any radiation damage mechanisms to the structure are not recorded. Several different biology and medical research groups find this type of microscopy particularly well-suited to the detailed analysis of sub-cellular features, and to the study of live organisms subjected to various forms of external stimuli. This technology utilizes bright x-ray sources produced by compact pulse laser systems. The incorporation of advanced x-ray optical and electron-optical systems will lead to the development of a compact, real-time x-ray microscope, having a broad range of applications.

## 1. INTRODUCTION

Microbiologists have at their disposal a range of imaging analytical instrumentation, ranging from an array of optical microscopies lead by recent advances in digital imaging, confocal and fluorescence microscopy, to the many forms of electron microscopy (SEM, TEM, PEEM, etc.). Each of these technologies brings unique capabilities, and many biological investigations are now based on observations by a number of separate imaging techniques. Although complex, all these microscopes are flexible, mobile systems. X-ray microscopy, is in principle capable of extending the spatial resolution of optical microscopy closer to that achieved with electron microscopy, while

preserving the ability to observe micro-organisms in their natural state. It has not yet reached this stage of development. Restrained for many years by the lack of x-ray optical elements and bright x-ray sources, it has existed more as a curiosity than as a viable observational tool. With the development of multilayer x-ray optics and precision metallic Fresnel lenses, and the construction of large second-generation synchrotrons, this situation has radically changed. Spectacular advances have been made in demonstrating the unique capabilities of x-ray microscopy. Its inherent superiority over optical microscopy in resolution is of major importance to studies of microscopic organisms. It also avoids the destructive preparation procedures necessary for electron microscopy. As a consequence there are now strong research programs directed towards the development of x-ray microscopic techniques for biological science. In this country they are all based on the use of synchrotron radiation generated at major national laboratories [1].

Research at synchrotron facilities has shown that x-ray microscopy fills a vital analytical gap between optical and electron techniques. Optical microscopy offers the ability to observe live organisms, in their natural environment, with the limited spatial resolutions (confocal) of ~ 200 nm, and depths of field of ~ 300 nm. These depths of field allow for the visualization of whole cell structures and phenomena, but are limited by resolutions unable to depict fine cell structure. Electron microscopy (SEM) offers high resolutions of ~ 1 nm, but small depths of field ( ~ 1 nm). The price for this high resolution is elaborate preparation of the specimen that includes: drying, staining, sectioning, and metallic coating the specimen. This type of preparation eliminates the possibility of observing live specimens, in a natural environment. The small depth of field typical in electron techniques eliminates the ability to view whole cell phenomena. X-ray microscopy, with resolutions of ~10 nm, and depths of field > 1 μm, bridges the gap nicely. X-rays offer high enough resolution to view the fine cell structure, without the morphology altering preparation involved in electron techniques. This method delivers, for the first time, the combination of live, whole cell, in vitro, observation with high resolution. In addition, the ability of x-rays to penetrate organic matter makes available the imaging of internal cell structure. Today's biologist makes use of all these techniques, each with its own merits, to give them an overall or "global imaging" view of the micro-organic world.

The heavy investment, and research that went into the development of x-ray techniques has lead to some spectacular advances in the deployment of state-of-the-art x-ray optical elements, particularly zone-plate lenses, in sophisticated x-ray microscopes on dedicated beamlines at major synchrotron facilities [2,3,4]. This intense activity has resulted in the development of several approaches to x-ray microscopy including scanning transmission [5], scanning phase contrast [6], and scanning dark field [7], and their use for high resolution imaging [8] of biological specimens in their natural and dried state, with significant progress being made towards intriguing options such as elemental and chemical mapping at the sub-cellular level [9,10]. There are however, some insurmountable drawbacks to this technological direction. X-ray microscopes attached to a beamline of a major national accelerator facility will never become a mobile, user-friendly device available at short notice for diagnostic analysis. It is also unlikely that this technical path will lead to the design of compact devices that become the seed from which corporate R&D takes the lead in developing this technology. Moreover, we have found that many biologists rely on analysis from several different microscopes, (confocal, fluorescence, electron, etc.) Easy access and proximity to other instruments becomes an important logistical requirement of an x-ray microscope technology. Another technical limitation stems from the fact that with current synchrotron-based x-ray microscopies it takes many seconds of x-ray exposure to capture an image, during which time the damaging effects of the x-rays to the biological structure of the organism can be registered on the image of the specimen. Various measures have been devised to mitigate this effect, including the use of chemical fixation, specimen dehydration [11] or fast freezing [12]. With the exception of the latter, all these measures are precisely those limitations of electron microscopy for biology that x-ray microscopy was meant to avoid. A larger long-term penalty of long exposure times is the inability to study fast kinetics in biological structures, such as the response of an organism to some specific physical or chemical stimulus or the interaction of two or more separate biological organisms.

The drawbacks associated with the use of synchrotron x-ray sources, has led to a search for alternate sources of x-rays. Laser-produced-plasmas can emit short pulses, (< 10 ns), of tunable, bright, (> $4x10^8$ W), of x-rays. Furthermore, with the development of high power, tabletop lasers, these systems are modular, and have the potential for development of a stand-alone device. In this paper, we describe progress in the development of this type of x-ray source, and its application to x-ray microscopy for biology and medical sciences. In particular we gave some examples of recent data obtained from a dedicated micro-radiography facility, and indicate some of the practical applications of this type of x-ray microscopy. Secondly, we outline a development pathway for a laser-plasma x-ray source based microscopy that produces images in real time. This system depends on the use of novel, high-

resolution x-ray optical components and a special x-ray opto-electronic image tube. We believe that this is the optimum architecture for a stand-alone mobile microscopic system.

## 2.0 A LASER PLASMA X-RAY MICROSCOPY FACILITY FOR BIOLOGISTS

We have deployed a laser plasma x-ray microscope facility that is both user-friendly and useful to the biological community, in order to demonstrate that x-ray microscopy, based on compact modular systems is a viable alternative to those based on large synchrotrons. Our strategy was to build a reliable facility based on projection micro-radiography. This approach, perhaps the oldest used for x-ray microscopy [13, 14] provides the high spatial resolution (~ 10 nm) required for small samples with a relatively compact and flexible system. Its principal disadvantages are that it employs 'line of sight' or projection imaging which suffers loss of resolution with thick (i.e. > 100 nm) specimens. Secondly, since the image is recorded with unity magnification, the resolution is set by the grain-size or pixel size of the recording media. In practice this means resorting to high resolution photo-resists as recording media, these having a grain size of ~ 5 nm (PMMA), since x-ray film and active array detectors have effective pixel sizes many times larger than this. Several methods have been devised to read the image registered within the resist. These all require chemical development of the resist, a somewhat variable procedure, and the subsequent analysis of the three-dimensional imprint of the image in the resist. Several methods can be used for the latter, including the use of a SEM, a TEM [15], and atomic force microscopy [16]. To date, the use of an AFM is the least ambiguous approach, and is relatively easy to implement. Nonetheless, each image requires several hours of specialized resist processing, drying and analysis. Notwithstanding these limitations, this facility provides the biological community with a flexible, open, x-ray microscope that is receiving increasing interest in the biology community.

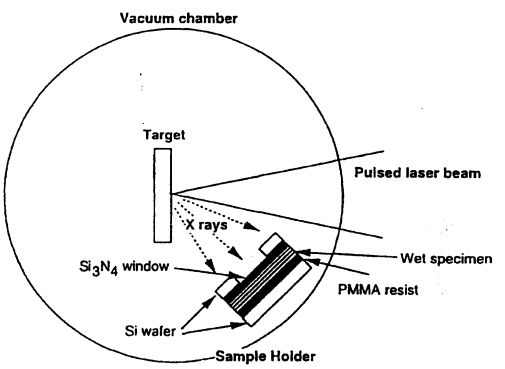


Fig.1 Schematic arrangement of laser plasma x-ray micro-radiography system

The principal elements of this facility are shown in Fig.1. The biological samples are loaded into a special, vacuum-sealed sample cell in their natural fluid. The sample cell has a thickness of ~10 μm. The samples are encapsulated between the PMMA photo-resist, which is deposited on a Si substrate on one side, and a thin (~100 nm) SiN window on the other side. The overall exposed area is a few square millimeters. The cell is exposed to one single burst of x-rays for a laser plasma source in a configuration shown schematically in Fig.1. The vacuum-sealed cell is located ~ 2 cm from the plasma source, which is a small ~100 μm diameter high-density plasma created from an Y or Au metal target. The plasma is produced by a ~10 ns burst of focused 1064 nm laser radiation having a total energy of 6 –20 J, generated from a Nd;glass laser system comprising a seed-injected, single-mode Q-switched oscillator followed by a passive four-pass amplifier incorporating an SBS phase conjugate mirror, and two additional linear amplifiers. The plasmas produced by this laser emit x-rays characteristic of a Planckian source with a temperature of ~ 100 eV, together with broad band N shell emission emanating from multiple-ion transitions in the 1-5 nm range. This is close to the so-called "water window" region, (2.3 – 4.5 nm) in which a strong contrast in ax-ray absorption (close to a factor of ~10) exists between protein material containing C and N, and the typical hydrogenous fluids in which they are contained. For the current conditions the conversion efficiency of laser light

into x-rays in this region is ~ 10%. Thus the number of x-ray photons incident on each square 10 nm pixel of the photo-resist is ~ 30 photons/ J of absorbed laser energy.

## 3.0 APPLICATIONS IN BIOLOGY AND NANOSTRUCTURES

This facility has been used to examine a variety of biological and physical structures. Below we illustrate the features and some of the advantages of our approach to x-ray microscopy.

The spatial resolution of this form of microscopy is demonstrated by the image shown in Fig.2, which shows an image of the outer region of a macrophage cell, immersed in a saline solution. The outer tendrils of the macrophage, having a diameter of less than 50 nm are clearly identified. In fact the resolution or our current system is limited solely by the present configuration of the AFM that is used to read the photo-resist. Earlier measurements of in-vitro human chromosomes demonstrated a resolution approaching 10 nm[17]. This is well beyond the resolution of an optical microscope. Moreover features such as the tendrils shown in Fig.2, although resolvable with electron microscopy, could not have been observed in their natural state, freely attached to a living organism immediately prior to the x-ray image was recorded.

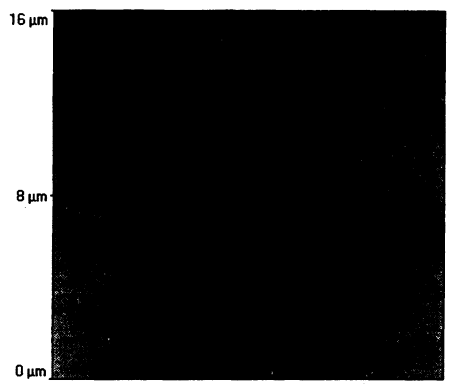


Fig.2. X-ray micro-radiograph of macrophage

Another principal advantage of x-ray microscopy based on laser plasmas derives from its capability to capture an image with a single burst of x-rays from the sources. This allows for an image to be registered in the resist before any biological, chemical or kinetic change can occur within the organism as a consequence of absorption of the x-rays. X-ray microscopy based on synchrotron sources cannot avoid this problem at present with room-temperature organisms in their natural state. Moreover, the use of short bursts of x-rays permits time-dependent studies to be made of transient phenomena in live organic specimens. Many of the users of the CREOL facility have expressed interest in, and the potential for time dependent studies with x-ray microscopy. An example of a recent study is shown in Fig.3. This shows an x-ray micrograph of a cell of *Pseudomonus aeruginosa* before and after it had been exposed to the drug, Gentamicin. Considerable changes in shape, and in the structure of the outside structure of the cell are observable. Other groups are interested in observing kinetic changes in specimens, such as motion in live muscle tissue, and the effects of radiation on specimens.

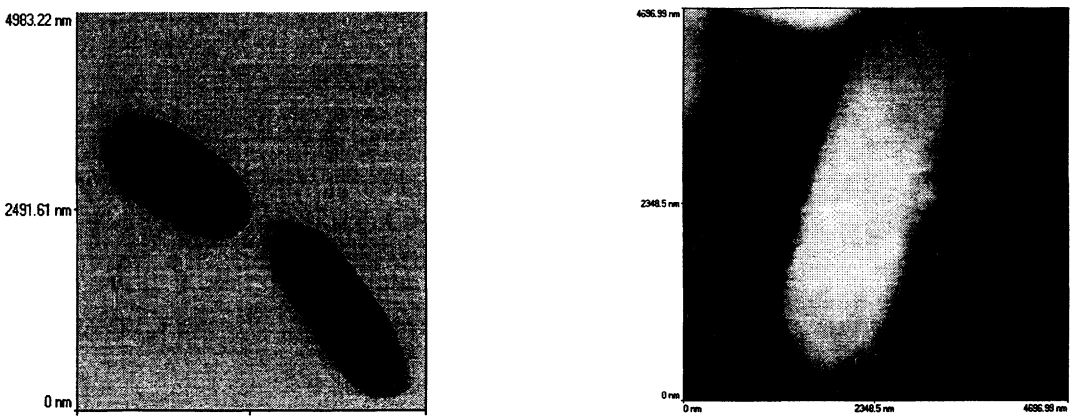


Fig.3. Timed x-ray micrographs of *Pseudomonus aeruginosa* under the effects of Gentamicin.

Time resolved microscopy can also be used to examine the effect of one organism on another. As an example, Fig.4. shows an x-ray micrograph of the interaction between peritoneal macrophage cells from a mouse and *Candida albicanas*, a pathogenic yeast, which causes candidiasis of immuno-compromised patients. Time-resolved x-ray microscopy is the only observational microscopy that has the resolution to observe the sub-cellular features on the nanometer scale in live specimens in this type of interaction.

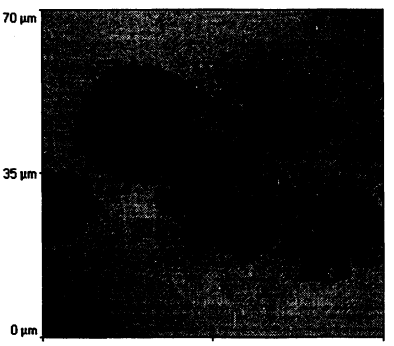


Fig. 4. Interaction of macrophage cells with *Candida albicanas*

Other features of laser-plasma based x-ray microscopy that are attractive for micro-biological studies include the possibility of elemental analysis, possible approaches to topographic imaging, and the potential for short-pulse laser-dependent probe or micro-interaction studies.

## 4.0 DEVELOPMENT OF A REAL-TIME, STAND ALONE X-RAY IMAGING MICROSCOPE

In order to extend the capabilities of our current system, we are developing a new kind of x-ray microscope. This microscope will have three principal advantages over our existing system. Firstly it will provide real-time imaging, without the requirement of post-shot processing procedures and delays. Secondly, this system will incorporate x-ray imaging optics, eliminating the problems of line-of-sight recording associated with the current radiographic approach, and allowing thicker specimens to be analyzed. Finally, the system architecture will be changed to facilitate a separate specimen chamber, amenable to changing the specimen environment, introducing external stimuli, and rapid specimen insertion for time resolved studies.

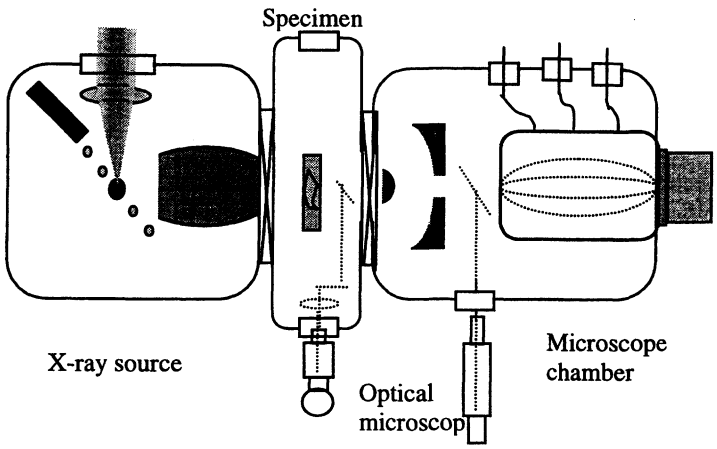


Fig. 5. Design features of a real-time compact x-ray microscope

This microscope, depicted in Fig 5, will draw upon several different technologies that have not previously been linked together in this way. The x-ray light source will be a compact laser plasma light source of a type we have recently developed and patented [18,19], that eliminates the plasma debris, a potentially harmful attribute of all other pulsed x-ray sources. The optical condenser section of the microscope will incorporate a novel form of x-ray collimator [20,21]. The specimen holder will be flexible in design, to allow adaptation of the microscope to dynamic biological studies and will include a co-axial optical microscope for precise specimen registration. The design of the imaging section of the microscope will depart from all previously used concepts for high-resolution x-ray imaging. As a first stage it will use a high numerical aperture, high-resolution, two element Schwarzschild optic [22, 23]. The image from this optic will be displayed with modest magnification onto the photocathode of a novel x-ray sensitive electron-optical image magnifier [24, 25, 26, 27, 28]. This precision electron tube will both magnify and intensify the electron image before converting it, via an phosphor converter to an image recorded by a high-resolution CCD that is the processed by conventional image processing techniques. The overall resolution will be set by the imaging x-ray optic, expected to be in the range of 30 nm. The time resolution of the instrument will, in its shortest mode of operation be in the range of ~ 100 ps, the shortest duration of the x-ray burst from a single laser pulse. We calculate that the instrument should detect single x-ray photons per pixel in the image plane. The overall image magnification will be 1000-10,000. The spectrum of the recording radiation will initially be set by the specifications of the imaging optic. However in a second phase of the development, we expect to change the x-ray optics of the instrument to allow x-rays over a broad range of energies to be recorded.

## 5.0 SUMMARY

In the project described above, we have demonstrated the use and potential of x-ray microscopy based on single shot laser-plasma x-ray sources. We have shown how this approach to x-ray microscopy has some advantages over those using x-ray radiation from synchrotron sources. We have formed a diverse Users Group to exploit this technology. Microbiologists, medical scientists and organic chemists are finding important applications of this type of microscopy. We are now in the process of developing the next generation of this type of microscope that will incorporate real-time imaging and quick, easy access for time-dependent analysis of live organisms. In the future we foresee that this system being amenable to commercial development into system that would provide biology and medical science, and other disciplines with a new diagnostic tool for microstructure analysis.

This work is supported by AFOSR (contracts F49620-94-1-0371 and F49620-93-1-0148), by NSF (contract ECS-9412008), and by the State of Florida.

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